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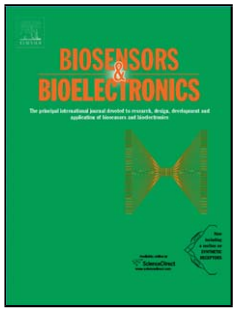

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Development of an Electrochemical Immunosensor for Aflatoxin M_1 in Milk with Focus on Matrix Interference

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Abstract

A simple sensor method was developed for aflatoxin M_1 analysis to be applied directly with milk by using antibody modified screen-printed carbon working electrode with carbon counter and silver-silver chloride pseudo-reference electrode. A competitive ELISA assay format was constructed on the surface of the working electrode using 3,3,5',5'-tetramethylbenzidine dihyrochloride (TMB) /H_2O_2 electrochemical detection scheme with horseradish peroxidase (HRP) as the enzyme label. The performance of the assay and the sensor was optimised and characterised in pure buffer conditions before applying to milk samples. Extensive interference to the electroanalytical signal was observed upon the analysis of milk. Through a series of chemical fractionations of the milk, and testing the electrochemical properties of the fractions, the interference was attributed to whey proteins with focus towards α-lactalbumin. A simple pre-treatment technique of incorporating 18 mM calcium chloride, in the form of Dulbucco's PBS, in a 1:1 ratio to the milk sample or standards and also to the washing buffer stabilised the whey proteins in solution and eliminate the interfering signal. The resulting immunosensor was interference free and achieved a limit of detection of 39 ng l^{-1} with a linear dynamic detection range up to 1000 ng l^{-1}. The developed immunosensor method was compared to a commercial ELISA kit and an in-house HPLC method. The immunsensor was comparable, in term of sensitivity, but vastly superior in term of portability and cost therefore a key instrument for the detection of aflatoxin M_1 at the source of the contamination.

Keywords: Immunosensor, Aflatoxin M1, Mycotoxins, Milk

1. Introduction

Although the first reported cases of mycotoxicoses was in 1722, not until 1960 was there significant research into the causes of mycotoxicoses with the onset of 'turkey X' disease (Farrer, 1987). At that time the mould *Aspergillus flavus* was isolated and correlated with aflatoxin production. Although *A. flavus* can grow in range of temperatures (10^{o}C - 45^{o}C), the optimum temperature is 30^{o}C. Additionally a relative humidity of 80% is required hence aflatoxin contamination is more of a concern in humid tropics regions (Moreau, 1979). It was also recognised that ruminants upon the consumption of aflatoxin B_1 contaminated feed would excrete aflatoxin M_1 through milk (Sargeant, 1961; Holzapfel & Steyn, 1966). Subsequently it has been shown that alfatoxin B_1 can also be produced to a lesser extent by *A. parasiticus*. It has been postulated that aflatoxin M_1 is a detoxification product of aflatoxin B_1 since the carcinogenicity of aflatoxin M_1 is lower than aflatoxin B_1 (Neal *et al.*, 1998). However, aflatoxin M_1 is still regarded as; carcinogenic, genotoxic, teratogenic and immunosuppressive compound. Reports have hypothesised that the excretion of aflatoxin M_1 is between 1 to 4% of the amount of ingested aflatoxin B_1 for cows milk (van Egmond, 1983).

Alfatoxin M_1 can be found in dairy based products such as cheese, yogurt and infant formulae (van Egmond, 1983; Sharman *et al.*, 1989; Martins and Martins, 2002), and also in human breast milk and acts as a good biomarker, (El-Nezami *et al.*, 1995). Due to the fact that milk intake in infants is high and when young they are vulnerable to toxins, the European Commission regulation 472/2002 imposes maximum permissible levels of aflatoxin M_1 in milk of 50 ng l^{-1} and 25 ng l^{-1} for infant formulae (Henry *et al.*, 2001; Gilbert and Vargas, 2003). Austria and Switzerland have imposed stricter limits of 10 ng l^{-1} whereas the USA have higher regulatory of 500 ng l^{-1}. Although most concerning is many underdeveloped countries do not impose aflatoxin M_1 restrictions.

The official methods of analysis for aflatoxin M_1 rely upon high performance liquid chromatography (HPLC) or thin layer chromatography (TLC) (Sydenham and Shephard, 1996) with sample extraction and clean up conducted before the analysis. Immunochemical techniques are becoming very popular for mycotoxins analysis with

many literature reporting the use of either a commercially developed enzyme linked immunosorbant assay (ELISA) or self developed immunoassays (El-Nezami, 1995; Thirumala-Devi, 2002; Lopez *et al.*, 2003; Rodriguez Velasco *et al.*, 2003; Rastogi *et al.*, 2004; Sarimehmetoglu *et al.,* 2004; Logrieco *et al*., 2004). Additionally liquid chromatography- mass spectrometry (LC-MS) (Sørensen and Elbæk, 2005) has also been employed. All of these methods are slow and most are performed in laboratory settings and by qualified personnel. Unfortunately the regions of the world which are most affected by aflatoxin contamination tends to be poorer areas with minimal laboratory facilities. In India, for example, a recent survey found that 87.3% of the milk based samples analysed were contaminated, of these 99% were outside European limits. This is a major concern considering that India is the largest producer of milk in the world (Rastogi *et al*., 2004). Therefore as stipulated by the united nations '*there is an urgent need for simple, robust, low-cost analysis methods, for the major mycotoxins, which can be used in developing countries laboratories*' (Proctor, 1994). Furthermore the United Nations are quoted saying that '*the systematic and complete monitoring of aflatoxin is a major challenge for the future, as food production increases* (Stroka and Anklam, 2002).

In this paper we present a cost effective, disposable immunosensor for the detection of aflatoxin M_1 which can be preformed in the field to meet the detection requirements set out by the European Union and fulfilling the requirements quoted by the United Nations. Primarily, the two main enzyme substrates used for immunosensors are alkaline phosphatase and horseradish peroxidase. Volpe *et al.* (1998) has reported that using 3,3',5,5'- tetramethylbenzidine, (TMB) as an enzyme substrate for horseradish peroxidase yield greater sensitivity than substrates for alkaline phosphatase. Furthermore with the designed immunosensor to be used in raw milk, naturally present alkaline phosphatase potentially may cause interference. Using TMB as a substrate is re-enforced by Fanjul Bolado *et al.* (2005) who reported that TMB out performs 2,2'-azino-bis-(3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS) and *o*-phenylenediamine (OPD), furthermore OPD and ABTS have shown to be mutagenic and carcinogenic (Voogd *et al*., 1980).

The oxidation of TMB is a two step reaction. Firstly the addition of hydrogen peroxide to heme group containing HRP enzyme, reduces the HRP to form an

intermediate (compound 1), involving a 2 electron process, by changing the heme (Fe^{3+}) group into a ferryl oxo iron (Fe^{4+}=O) and a porphyrin (P) cation radical. Upon the addition of TMB, 2 molecules of TMB are oxidised by compound 1 to form a blue coloured electrochemical product. Upon the release of H_2O the peroxidase returns to the native state via a further intermediate, leaving the TMB in an oxidized state. Commonly sulphuric acid is added to the oxidised TMB to develop a stable yellow diiamine product that is measured at 450 nm and can be measured by differential pulsed voltammetry (Josephy *et al.*, 1982; Ruzgas *et al.*, 1996; Frey *et al.*, 2000; Tanaka *et al*., 2003).

In this work we report the development of a screen- printed electrode immunosensor, based on a competitive reaction between the free aflatoxin M_1 in the sample and an aflatoxin M_1 – horseradish peroxidase conjugate, for an immobilised monoclonal antibody for aflatoxin M_1. Using chronoamperometry, the signal generated by the use of TMB / H_2O_2 was monitored to ascertain the concentration of HRP on the sensor and consequently the concentration of aflatoxin M_1 in the sample. The immunosensor was optimised with regard interferences from the milk matrix. The simple method of milk sample pre-treatment which was developed in this work and combined with the optimised sensor is novel and being reported for the first time in this application.

2. Material and methods

2.1 Reagents and solutions

Aflatoxin M_1 was purchased from Axxora UK Limited (Nottingham, UK), Anti-aflatoxin M_1 antibody (raised from rat) from Abcam Limited, (Cambridge UK), Aflatoxin M_1-HRP conjugate from a RIDASCREEN® kit from R-Biopharm (Glasgow, UK) as well as Alfaprep® M immunoaffinity columns. 3,3',5,5'-Tetramethylbenzidine dihydrochloride, hydrogen peroxide, fish skin gelatine, polyvinyl alcohol, polyvinylpyrrolidone and Tween 20 purchased from Sigma-Aldrich (Poole, UK). Anti-Rat immunopure antibody (raised in goat with affinity for the Fc fragment only) was from Perbio Science (Cramlington, UK). Milinex sheets from Cadillac plastics (Swindon, UK), Electrodag 423-SS graphite ink, Electrodag 6038-SS Ag/AgCl from Acheson industries (Plymouth, UK), Blue epoxy insulating

ink 242-SB, from ESL electroscience products (Reading, UK), Milk and dried milk samples were obtained from the local supermarket.

2.2 Electrodes Fabrication

Screen- printed electrodes (SPEs) were fabricated in house by a multistage deposition process using a DEK 248-screen printer and stencils (DEK, Weymouth, UK) (Kadara and Tothill, 2004). The electrodes were printed using 250 μm thick polyester Melinex sheets. The print parameters were set so that the squeegee pressure was 4 psi, a carriage speed of 50 mm sec^{-1} and a print gap of 2.5 mm. For the fabrication, the basal tracks for the three-electrode system were printed first using Electrodag 423-SS graphite ink. The reference electrode was printed on one of the basal tracks using Electrodag 6038-SS silver-sliver chloride ink and left to dry. The two other tracks (graphite-carbon working electrode with a 5 mm diameter giving a 19.6 mm^2 planar area and a graphite carbon counter electrode (1.3 mm^2 planar area). The blue epoxy insulating layer was printed last using 242-SB protective polymer. Between each layer the sheets were allowed to dry for 2 hours at 60^oC and then after the insulating layer the sheets were cured at 120^oC for two hours. The different inks used and the polyester sheet used in the sensor fabrications are stable at this temperature.

2.3 Procedures

Electrochemical measurements

For the electrochemical procedures a computer controlled four channel Autolab electrochemical analyser multipotentiostat (Eco Chemie, Utrecht, The Netherlands) was used throughout which allows the simultaneous detection of four sensors. Data capture was through the supplied GPES version 4.9 software installed onto a PC. The screen-printed electrodes were connected to the Autolab, using an in house fabricated connector from a PCB edged IDC socket, aluminum instrument box, ribbon cable and 4 mm cable sockets. The individual components were purchased from Maplin Electronics (Milton Keynes, UK). For the C.V. scans a 100 μl of sample drop was placed onto the electrode and was disposed of after each scan. The scanning range was from -1 to +1 V at a rate of 99.78 mV/s with steps of 2.74 mV. Studies into the suppression effects of milk used samples of milk with different pre-treatments mixed with 5 mM potassium hexacyanoferrate (III) in 0.1 M KCl.

Immunoassay developments

For the sensor construction, 8 µl of 0.12 mg ml^{-1} anti-primary antibody in 0.1 M carbonate buffer pH 9.6 was placed onto the working graphite electrode, placed into a humid environment (stored overnight at 4^{o}C), to allow passive adsorption of the antibody onto the carbon surface. The sensor was then washed with 0.05 % Tween 20 in 10 mM PBS buffer and 18.0 MΩ water. The electrodes were then shaken to remove most of the surplus water and anti-aflatoxin M_1 monoclonal antibody at 0.04 mg ml^{-1} (8 µl) in 10 mM PBS buffer was added and incubated for 2 h at 37^{o}C, in a humid environment. The surface of the sensor was then blocked by immersed in 1 % PVA in PBS to cover the working, reference and auxiliary electrodes for 2 h at 37^{o}C. The sensor was then washed and stored at 4 ^{o}C until used.

Aflatoxin M_1 standards were prepared by dissolving the aflatoxin M_1 powder in methanol at a concentration of 10 mg ml^{-1} to prepare a stock solution and then stored at –18 ^{o}C. Working standard solutions (between 5 and 1000 ng l^{-1}) were prepared by diluting the stock with 1% methanol in 20 mM Dulbecco's PBS ($CaCl_2$ concentration of 18 mM) pH 7.4, into twice the desired concentration and then mixing 500 µl of standard with 500µl of commercial milk. Milk samples were also pre-treated by adding 25 ml of 20 mM Dulbecco's PBS ($CaCl_2$ concentration of 18 mM) pH 7.4, in 1% methanol to 25 ml of milk sample and mixing. This was carried out using a vortex mixer.

For the competitive reaction a 4 µl of aflatoxin M_1 standard or sample was diluted in PBS buffer with 1% methanol, and placed onto the working electrode with 4 µl of 1:10 dilution of the aflatoxin M_1-HRP conjugate from the RIDASCREEN® kit diluted using 1% PVA in PBS. No specific sampling protocols were implemented for milk sampling since milk is considered homogeneous (van Egmond, 1983).

The competitive reaction between the free aflatoxin M_1 and the aflatoxin M_1 – HRP was performed at 37^{o}C for 2 hours. The sensor was again washed, shaken to almost dryness, then 100 µl of 5 mM 3,3′,5,5′-Tetramethylbenzidine (TMB) and 1 mM hydrogen peroxide in citrate buffer containing 0.1 M KCl, was added to the sensor

ensuring all three electrodes were covered. The Autolab running in chronoamperometry mode was started and the data collected for 20 minutes. For the chronoamperometry data points were collected every 2 seconds at a potential of either -100 mV or +100 mV. For electrode preconditioning a conditioning potential of +200 mV was applied for 20 seconds followed by an equilibrium time of 5 seconds before data was collected at +100 mV.

Step amperometry was performed by adding 10 units of horseradish peroxidase, to a solution of 5 mM TMB and 1 mM hydrogen peroxide in 0.1M KCl citrate buffer, then incubating for 30 minutes before measurement. A blank signal was obtained without the addition of peroxidase. The Autolab was set for steps of 100 mV from 0 mV to either -900 or +900 mV and current measurement for 100 seconds.

For the fractionation of the casein and whey proteins of milk, a similar method to that described by Vernozy-Rozand *et al.*, (2004) was implemented. Firstly a commercial whole fat milk sample was initially centrifuged at 9600 x g to remove the cream and fatty layers. The supernatant was decanted and adjusted to pH 4.6 with the use of 4 M hydrochloric acid, stirred for 30 minutes and then centrifuged again to obtain casein free liquor. For the removal of whey proteins the supernatant was treated with 5 M trichloroacetic acid and stirred for 30 minutes before centrifugation. The remaining liquor was free from proteins.

Calculations of limits of detection for the immunosensor was determined as described by Ammidia *et al*., (2004) and Draisci *et al*., (2001) as the amount of aflatoxin M_1 required to reduce the signal change by 25%.

2.4 HPLC Analysis

The in-house HPLC determination was performed using a Waters 600E System Controller, a Waters 712 WISP Autosampler and a Waters 470 Scanning Fluorescence Detector set at an excitation wavelength of 360 nm and an emission wavelength of 430 nm. The Waters modules were computer controlled using Kromasystem 2000 software. A Phenomenex Luna 5u C18 analytical column was used throughout with a security guard TM guard column. Aflatoxin M_1 standards were made up with 1% methanol, 49% of 20 mM, pH 7.4, PBS buffer and 50% milk

sample. The toxin was then extracted from the milk samples using Alfaprep® immunoaffinity columns as denoted by the manufactures R-Biopharm. Briefly 50 ml of spiked milk was centrifuged at 3,000 RPM to isolate the fat and then passed through the immunoaffinity column at a rate of 1-2 drops per second. The column was washed with 2 aliquots of 10 ml H_2O and eluted into a eppendorf tube with 1.25 ml of 2:3 methanol:acetonitrile followed by 1.25 ml of H_2O. After mixing by vortex, the sample was divided into three and placed into HPLC vials for triplicate analysis.

2.5 Safety awareness

All laboratory glassware and consumables which had been contaminated with aflatoxin M_1 was stored overnight in 5% sodium hypochlorite followed by the addition of acetone to make the solution 5% acetone by volume. The decontamination solution was allowed a minimum of 30 minutes before disposal.

3. Results and Discussions

3.1 Optimisation of the immunosensor

For the immunosensor developments TMB was chosen as the mediator for the enzyme label, horseradish peroxidase (HRP) activity determination. Previous work at Cranfield has been preformed using hydroquinone and *o*-phenylenediamine (OPD) as the mediators for hydrogen peroxide (Baskeyfield, 2001). The application for the sensor is for point of source monitoring in field work, therefore the use of carcinogenic compounds is not preferable. Furthermore TMB has superior detection properties than other systems (Fanjul-Bolado *et al.*, 2005, Volpe *et al.*, 1998) .The initial protocol for the immunosensor development was adopted from Micheli *et al*. (2005). However, it was noticed that there are discrepancies in the literature into the optimum potential for the electrochemical detection of TMB using carbon electrodes. Micheli *et al.*, (2005) reported the detection of TMB at -100mV *versus* Ag/AgCl, whereas Butler *et al.*, (2006), Fanjul-Bolado *et al.,* (2005) and Volpe *et al.*, (1998) suggest a voltage at +100mV *versus* Ag/AgCl. Since no previous literature reports could be found where the preferential potential had been discussed, step amerometry was performed to elucidate the correct potential for the developed immunosensor.

Therefore a range of potentials from -900 mV to +900 mV were investigated using the developed screen-printed electrode. Figure 1, show that the best potential for monitoring the reduction was -100 mV and for the oxidation +100 mV. This is harmonious with the previous reported observations.

Figure 1

The step amperometry suggested that +100 mV would yield stronger signal to blank ratio than -100 mV. An additional experiment was preformed to validate this observation. Figure 2a, show that although the reduction signal gave a greater signal than the oxidation signal, it incurred a high blank signal, hence for the development of the sensor the oxidation signal was monitored. The use of electrochemical preconditioning of the electrode for immunosensor development has been reported recently (Conneely *et al*., 2007; Lu *et al.*, 2006). Therefore, to maximize the signal, the use of electrode pre-conditioning was investigated in this work. To precondition the electrode a conditioning potential of +200 mV was applied for 20 seconds before detection of TMB at +100 mV. Figure 2b shows that although there is little advantage with respect to the background levels, there is significant gain in signal by pre-conditioning the sensor before data collection. Further electrode treatment was investigated to depolarise the electrode surface before antibody immobilization (Grennan *et al.*, 2000; Espinosa *et al.*, 1999; Wang *et al.*, 1996). Summarising the literature, the use of a potential of 2.0 V from 30 seconds to 10 minutes was applied to increase protein immobilization capacity and electron-transfer rates of the working electrode, in turn increasing the signal and reproducibility. The same treatment was performed for our electrodes to deem if this treatment would increase or produce a more reproducible signal. As shown in Figure 2c, although the depolarisation did produce a greater signal, the difference is marginal. Additionally the cleaning resulted in a high standard deviation therefore considering the additional time incurred from depolarisation the electrodes it was deemed that this step was not fundamental to increasing the sensors performance. However, further testing may prove beneficial to elucidate this point in future work. The use of different blocking buffers with different chemistries was also investigated (Figure 2d). Using the screen- printed electrode, PVA wasfound to be the optimal blocker. PVPP (Polyvinyl pyrrolidone)

was also tested but yielded a high standard deviation and therefore was not used in this experiment (data not shown).

Figure 2

With the signal ameliorated a calibration curve was performed in pure buffer undertaking the factors from the optimisation experiments (Figure 3). The dynamic range from 1 to 10,000 ng l^{-1} possessed a linear r^2 value of 0.95.

Figure 3

Upon performing the calibration in a full fat milk sample with no pre-treatment the correlation between concentration of aflatoxin M_1 and current was lost. Previous reports from Pemberton *et al.* (1999) stated that electro-active species can interfere with the detection of progesterone in milk. Mayer *et al.* (1996) have reported that milk can cause electrode fouling without pre-treatment, but, upon dialysis with 12000-19000 molecular size cut off membranes then the matrix effects are removed. A cyclic voltamogram of TMB, with and without the addition of commercial full fat milk, was carried out (data not shown) and the milk suppressed the signal. To establish the cause of the interference several chemical clean up strategies were employed, and tested by monitoring the electrochemical quenching effect. To ascertain the effects of fats to the system a commercial milk sample (pH adjusted to 8.6) was incubated at 37^{o}C for 24 hours to activate the natural lipases and thus breaking down the fats into fatty acids (Hui, 1993) was used with a second non-fat milk sample (Sigma – Aldrich). Both samples quenched the electrochemical signal from potassium hexacyanoferrate, suggesting that fats are not the cause of the interference (Figure 4a).

Figure 4

Mayer *et al.* (1996), reported that lactose was an interfering compound for their milk based biosensor. Furthermore the electro active nature of lactose is taken advantage of as a method of detection using ion chromatography (Hanko and Rohrer, 2000). To determine the electrochemical effects of lactose, potassium hexacyanoferrate was spiked with 4.6% lactose to replicate the natural concentration in milk (Schrimshaw,

1988). Figure 4b, shows that lactose has no quenching effect, this is to be expected since lactose is below the molecular weight which Mayer *et al.* (1996) reported as being responsible for electrode fouling. Milk was then fractionated into a casein free sample (Hui, 1993; Walstra, 1984), and a casein and whey protein fraction (Vernozy-Rozand *et al*., 2004) as reported in the methods. By isolating the casein proteins, significant quenching still occurs, however, upon the removal of whey proteins, the signal was not affected (Figure 4c). To confirm this a milk sample was saturated with ammonium sulphate and stored at 4^{o}C for 48 hours, then centrifuged. The pre-treatment with ammonium sulphate removed all traces of the interference (the induced pH shift from ammonium acetate is the cause of the sharper peaks) confirming that the electrochemical interference from milk is due to a proteinaceous compound (Figure 4d). Whey proteins otherwise known as 'milk serum' proteins are a group containing; β-lactoglobulin (18,363 daltons), α-lactalbumin (14,176 Daltons) and bovine serum albumin (66,267 Daltons), additionally the groups also contains immunoglobins and small molecular weight peptides (Walstra 1984). The molecular weight of β-lactoglobulin, bovine serum albumin and α-lactalbumin correlates with the reports of Mayer *et al.*, (1996) that the electrode fouling was eradicated by the use of dialysis membranes at 12,000 – 19,000 daltons. Furthermore Diaz *et al.* (1993) advocates the use of dialysis membranes at 8,000 to 15,000 Daltons for the clean-up of milk for aflatoxin M_1 determination using TLC. Cosman *et al.*, (2005) reinforces this observation.

Cosman *et al.* (2005), reported that whey proteins spontaneously adsorbs onto metal surfaces through a variety of different chemistries. It was suspected that α-lactalbumin immobilization was due to the loss of calcium causing significant disruption to the protein structure and thus denaturation. From this observation an excess of calcium chloride (18mM) was added to the milk sample and also washing buffer during immunosensor analysis. The resultant effect was losing of the suppression and a detection limit of 39 ng l^{-1} was achieved in milk samples (Figure 5). The concentration of 18mM $CaCl_2$ was chosen to mimic that suggested by Dulbecco *et a*l. (1954) upon the work with the isolation of viruses. The recipe later became known as Dulbeccos PBS and is a standard buffer used for maintaining the structure of mammalian cells. This $CaCl_2$ concentration has been shown to have no effect on the antibodies activity.

Figure 5

The developed immunosensor method was compared to an in-house HPLC method developed for aflatoxin M_1 and a commercial ELISA kit for aflatoxin M_1 (R-Biopharm). Milk samples were prepared using the calcium chloride pre-treatment method developed in this work and the same sample was then analysed by all three methods. For HPLC analysis, the sample was then extracted using an immunoaffinity column. Figure 6, shows the calibration graphs for all three methods.

Figure 6

The plots in Figure 6 show the success of the immunosensor developed method. Compared to the ELISA procedure, the immunosensor has similar limits of detection and comparable repeatability although the working range of the immunosensor is far greater than the ELISA method. In comparison the HPLC was more sensitive than the immunosensor with a limit of detection of 10 ng l^{-1} for the HPLC *verses* 39 ng l^{-1} for the immunosensor based on a 3 times signal to noise ratio, but, with similar dynamic range from 10 to 1000 ng l^{-1} (r^2 value of 0.9944). However, the sample used for the HPLC analysis had to be first extracted and purified using an immunoaffinity column which makes the method more complex and expensive. Also the analysis has to be conducted under laboratory conditions, while the sensor is portable, simple and cost effective and can be used on site.

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4. Conclusions

In this work the development of an electrochemical immunosensor for aflatoxin M_1 analysis was developed using a disposable screen-printed electrode. After immobilising the immuno-components to the electrode surface and optimising the assay format, the effects of milk on the sensor was assessed. It was discovered that the milk matrix causes significant interference, and through chemical fractionation, it was noted that the interference was chiefly resulting from whey proteins. When an excess

of calcium chloride (18mM) was added to the milk sample, and to the washing buffer, the effect was suppressed and a working calibration down to 39 ng l^{-1} was obtained with linear detection range up to 1 μg l^{-1}. Therefore the use of calcium chloride to stabilise milk samples on metal electrodes is advised for future immunosensor developments.

The immunosensor is not solely suitable for milk, but initial investigations have shown that it could be employed for aflatoxin M_1 determination in urine also to monitor human aflatoxin M_1 consumption. Upon comparing the immunosensor to the established technologies of HPLC and ELISA, the immunosensor was unique in offering good sensitivity as well as total portability.

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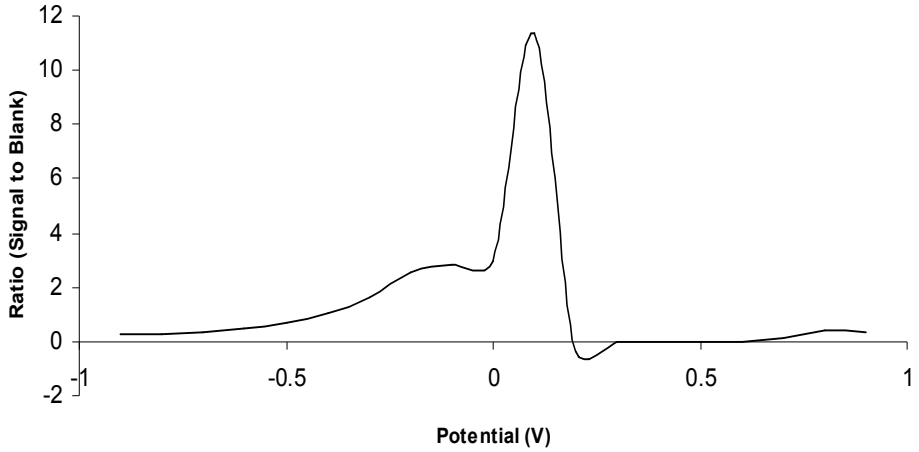

Figure 1: The ratio of the signal current to background current using step amperometry of 5 mM TMB/1 mM H_2O_2 with and without the addition of peroxidase in pH 5.2 citrate buffer, 0.1 M KCl. The data is a result from an average of 4 electrodes.

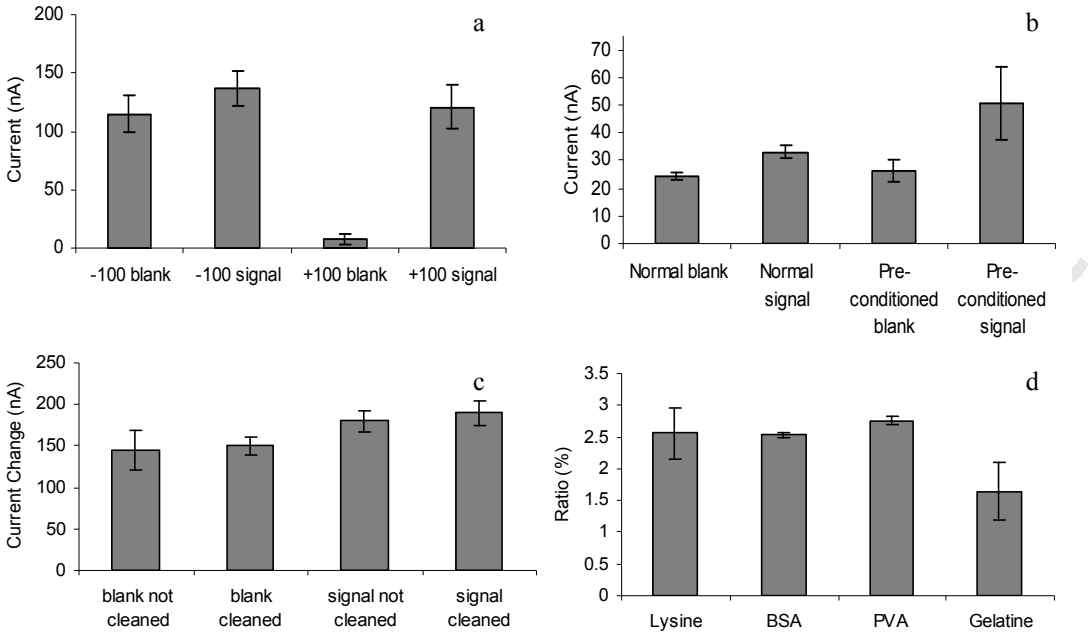

Figure 2: (a) Comparison of different sensing potentials. the blank comprised of the complete sensor system without the addition of aflatoxin M_1 – HRP conjugate. (b) Effect of electrode preconditioning, (the blank similar as above). Preconditioning was performed by applying a potential of +200 mV for 20 seconds followed by a five second equilibration stage before the data collection at an applied potential of +100 mV. (c) Electrodes were pre-cleaned with water, ethanol and then applying a potential of 0.8 V for 30 minutes with the electrode covered with PBS before the application of the anti-primary antibody. (d) Different blocking reagents (1% in PBS buffer), allowed to adsorb for 30 minutes at room temperature. Figure shows the ratio of the signal current and blank current where the blank signal was obtained using the complete sensor without the addition of aflatoxin M_1 – HRP. For all graphs error bars indicate the standard deviation (n=4).

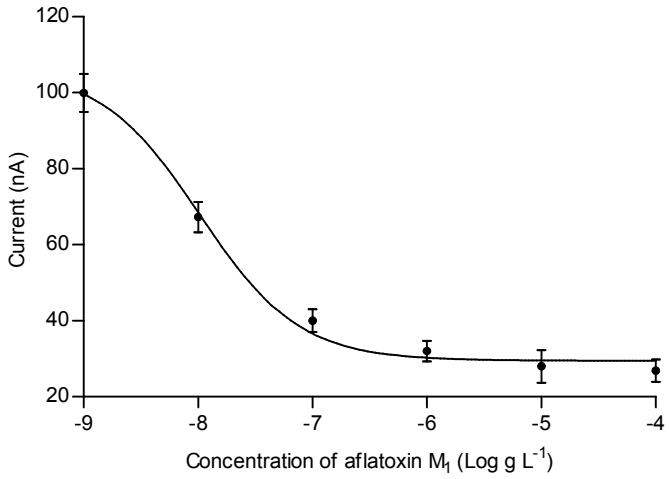

Figure. 3: Standard curve for the detection of aflatoxin M_1 using the electrochemical sensor. Signal was obtained using electrochemical preconditioning and data collection at a potential of +100 mV for 10 minutes. Error bars indicate the standard deviation (n=4). The dynamic range from 1 to 10,000 ng l^{-1} possessed a linear r^2 value of 0.95.

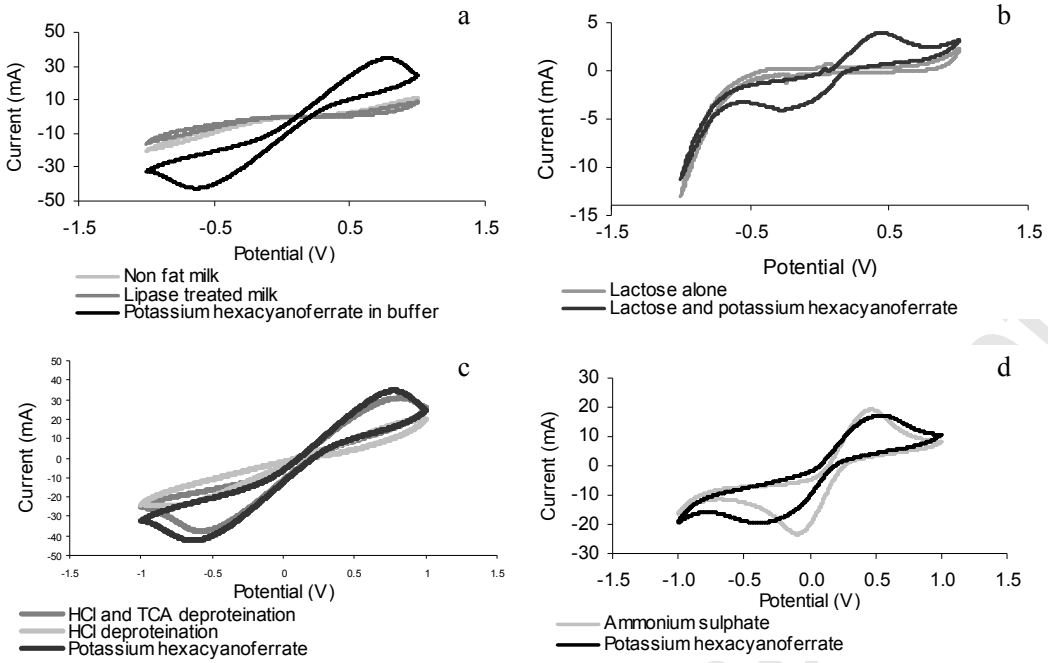

Figure 4: Cyclic voltammogram of potassium hexacyanoferrate (III) with and without the presence of (a) non-fat milk or milk subjected to natural activated lipases. (b) 4.6% lactose. (c) milk liquor subjected to deproteination with HCl and HCl/TCA. (d) deproteinated milk saturated with ammonium acetate.

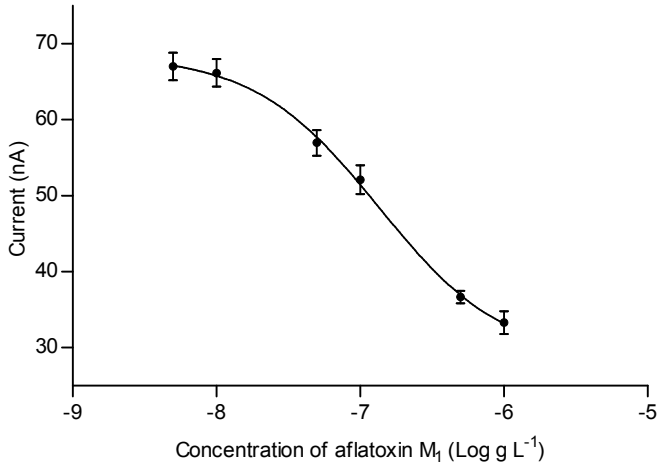

Figure 5: A calibration using calcium chloride for milk pre-treatment and fresh sensors. Error bars taken from standard deviations (n=3).

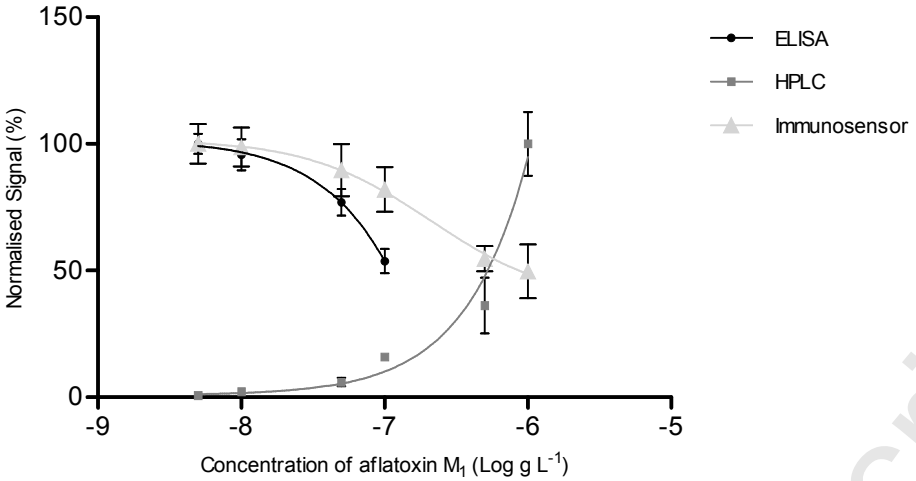

Figure 6: Comparison between the developed screen printed immunosensor against an in-house HPLC methods using immunoaffinity pre-treatment and a commercial R-Biopharm RIDASCREEN® ELISA kit. The same samples were used for all three methods and performed on the same day of analysis. For comparison the scale has been normalised to the highest signal for each method. Error bars denote standard deviations (n=3).